



The Biotechnology Education Company®

EDVOTEK®

284
EDVO-Kit #

Plant Pigment Chromatography and Photosynthesis

Storage:

See page 3 for specific storage requirements.

EXPERIMENT OBJECTIVE

The purpose of this experiment is to demonstrate light dependent reactions of photosynthesis by colorimetric assay and to gain an understanding of the biochemical processes by which they work. Through use of thin layer chromatography to separate plant pigments, students will gain an understanding of the theory and applications of chromatography.

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EDVO-Kit # 284 Plant Pigment Chromatography and Photosynthesis

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This experiment is designed for 10 lab groups

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Experiment Components

Store Components A - G in the Freezer

- A Chloroplast Extraction Buffer, concentrate, contains sorbitol, magnesium salts and is buffered at pH 7.6
- B Reducing Reagent
- C Chloroplast Dilution Buffer, concentrate, contains phosphate buffer, sorbitol and stabilizers
- D DPIP (2,6-dichlorophenol-indophenol), concentrate
- E Standard pigments (chlorophylls a+b, xanthophylls, β -carotene)
- F Chromatography solvent (A mixture of ethanol, isopropanol, and water with non-ionic polymer additives)
- G Sarkosyl, concentrated ionic detergent

Store Remaining Components at Room Temperature

- Thin layer cellulose chromatography plate
- Capillary pipets (5 μ l)
- Plastic transfer pipets
- Microtest tubes with attached lids
- Plastic centrifuge tubes (15 ml)

Requirements

- Visible wavelength spectrophotometer
- Pencils
- Clinical bench top centrifuge
- Metric rulers
- Test tube racks
- Flood light or small portable lamps (60 watt bulb is sufficient)
- Pipette pumps or bulbs
- Distilled water
- 13 x 100 mm (10 ml) test tubes
- 100 ml beakers
- 1 ml and 5 ml pipets
- Ice
- Cheesecloth
- 95-100% isopropanol* (2-propanol)
- Aluminum foil
- Fresh spinach
- Graph paper

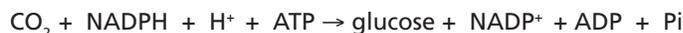
**Rubbing alcohol will not work since it is only 70% isopropanol.*

Background Information

Photosynthesis is the process by which plant cells use light energy for the biosynthesis of cellular components. Photosynthetic organisms form the basis of the food chain. These life forms include higher plants, algae, dinoflagellates, euglenoids, diatoms and certain bacteria. Photosynthesis consists of two biochemical phases. The general equation for the **first phase** is:



The first phase is **light dependent**. NADP⁺ and NADPH are oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide phosphate. The reduced form is an essential cofactor in the biosynthesis of many types of molecules such as carbohydrates. Chemical energy of ATP is required for many biochemical reactions and for maintenance of cellular integrity and function. ATP is generated from ADP and inorganic phosphate (Pi). The reaction also generates protons (H⁺) and molecular oxygen from water. The **second phase** of photosynthesis can be generally written as:



The second phase is **not light dependent**. The reaction fixes atmospheric carbon dioxide into organic linkage (glucose). Each phase consists of many separate chemical steps. First phase steps are called **light reactions** and second phase steps are called **dark reactions**.

Light reactions in eukaryotic cells occur in organelles called **chloroplasts**. Chloroplasts contain DNA and are self-replicating. These organelles consist of an outer membrane and a folded inner membrane. Stacked, disk-like structures called **thylakoids** form part of the inner membrane and it is here that light dependent photosynthetic systems are found.

The primary photosynthetic pigments are green chlorophylls. Chloroplasts contain chlorophyll 'a' and 'b', magnesium-porphyrin complexes, and are specifically bound to proteins that reside on and within the inner membrane. Pure **chlorophyll 'a'** maximally absorbs light at wavelengths of around 420 and 660 nm. **Chlorophyll 'b'** absorbs primarily at approximately 480 and 640 nm.

The absorption spectrum of chlorophylls can be shifted depending on with which type of protein they are associated. Other pigments found in chloroplasts include β-carotene and xanthophylls. These pigments have an accessory light harvesting function and absorb at wavelengths in between the maxima of the chlorophylls. They all capture light energy and transfer it to the chlorophyll *a* at the reaction center. All these pigments are sensitive to light and oxygen in the purified state and eventually breakdown. Extremely pure preparations required for chemical and biological studies are stored under vacuum, in the dark, at -20° C.



Background Information

PHOTOSYNTHETIC SYSTEMS

There are **two photosynthetic systems** in chloroplasts, termed **Photosystem I** and **II**. These physically distinct systems contain different proteins and ratios of chlorophylls and accessory pigments. **Photosystem I** is not responsible for oxygen evolution and is activated by longer wavelengths of light. **Photosystem II** is activated by shorter wavelengths of light and is required for oxygen and ATP production. Both systems contribute high energy electrons for the reduction of NADP⁺. Both photosystems are required for maximal photosynthetic activity. When light is absorbed by the chlorophyll-protein complex of photosystem I, chlorophyll becomes excited and enters a higher energy state. During return from high energy to ground state, an electron is boosted to a higher energy level and is sequentially transferred, via several membrane associated protein transport molecules, to the final **electron acceptor** NADP⁺. The NADP⁺ is reduced to NADPH through the action of a reductase enzyme. Since this is not normally a spontaneous (energetically favorable) reaction, input of light energy is required to convert NADP⁺ to NADPH.

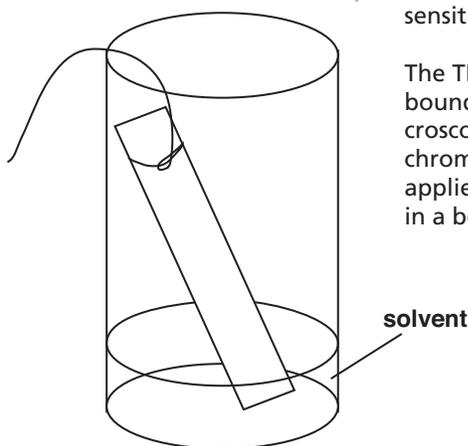
Electron transport proteins containing iron and sulfur are called **ferredoxins**. Other transporters are called **cytochromes**. A high energy electron generated by light absorption in photosystem II is donated, via a specific sequence of transporters, to the electron deficient photosystem I. Photosystem II then receives an electron through a series of transport proteins from H₂O. Water is oxidized to molecular oxygen during this process. Water is the electron donor in photosynthesis. Conversion of water, a very stable molecule, to oxygen is energetically unfavorable and would not occur to any significant extent without input of light energy at photosystem II. Production of ATP occurs along the sequence of electron transfer steps. ATP is a high energy compound and requires energy for its synthesis. This energy is siphoned from the high energy electrons through a complex series of events involving membrane proteins and formation of pH gradients across chloroplast membranes to drive ATP synthesis.

The **dark** reactions of photosynthesis occur simultaneously with the light reactions in plant cells. The dark reactions are a set of seven enzyme catalyzed metabolic steps that synthesize glucose. The key metabolic step in plant glucose synthesis is catalyzed by the abundant enzyme ribulose diphosphate carboxylase. The majority of these reactions take place outside the chloroplast in the cytoplasm. Most of the glucose is polymerized into starch and cellulose. The reaction involves the fixation of carbon dioxide.

Background Information

CHROMATOGRAPHIC BASICS

Thin layer chromatography (TLC) is a valuable method used for the separation and analysis of a wide variety of biological molecules. Separation of mixtures of lipids, amino acids, sugars, nucleotides or plant pigments is often accomplished with TLC methods. The separation procedure is generally very sensitive to small differences in chemical structure. The structure affects the strength and type of interactions between sample and adsorbent. The stronger the interaction, the slower the migration rate. In addition, different sample molecules will have different solubilities in a given solvent system based on their chemical structure. The separation of the pigments by TLC has many advantages over using paper chromatography. It is faster, it shows better resolving power and greater sensitivity, and it is more convenient to use.



The TLC plate typically consists of a very thin layer of adsorbent material bound to a glass or plastic support. The adsorbent consists of many microscopic edges, corners or fibers. These surfaces provide a large area for chromatographic separation. After a small volume of sample solution is applied to the adsorbent surface and allowed to dry, the plate is placed in a beaker or tank containing the appropriate solvent. Only the edge of the plate below the samples is in contact with the solvent. The solvent is drawn by **capillary action** into the dry adsorbent material and travels up the plate through the samples. The migration rate of the sample components through the adsorbent depends on their chemical structure.

An understanding of TLC first requires an introduction to **adsorption chromatography** which was discovered by the botanist Tswett in 1903. He observed that mixtures of plant pigments could be separated into different colored zones by passing them through a column of calcium carbonate. Significant development of adsorption chromatography occurred in the 1930's when it was used in the preparative chemistry of pigments. Other common adsorption chromatography materials include magnesium carbonate, silica gel, alumina and activated charcoal. Most adsorption materials are oxides, hydrated oxides or salts and can have surface charges. Substances that are adsorbed to these materials are **polar** or **polarizable** molecules. An example of a highly polar molecule is water. The unionized water molecule has no net charge. However, the oxygen atom has slightly more negative charge than the hydrogen atoms (which have slightly more positive charge). This is because the oxygen nucleus attracts the negatively charged electrons in the chemical bonds more strongly than the hydrogen nuclei.

Water molecules are overall electrically neutral but the individual atoms within a water molecule possess partial negative

Solvent	
Front	
Xanthophyll	
Chlorophyll b	
Chlorophyll a	
β -carotene	
Pigments at the origin	



Background Information

Background Information

(the oxygen) or positive charge (the hydrogens) Molecules that exhibit partial or net charge possess a **dipole**. Adsorbent materials are polar and fully possess charged chemical groups on their surface that can interact with these groups by dipole-dipole and hydrogen bond interactions. These interactions basically involve the electrical attraction between regions of opposite partial charges. By contrast hydrogen bonding involves the attraction of hydrogen atoms that form ionic bonds with sulfur, oxygen, nitrogen, or fluorine, to other electronegative atoms (typically oxygen or nitrogen atoms that make up other molecules or more distal regions of the same molecule). Many molecules have a net negative or positive charge and can interact with groups having the net opposite charge on the adsorbent surface. These electrostatic attractions are ion-ion interactions and form the basis of ion-exchange chromatography.

Partition chromatography is based on solubility differences exhibited by sample molecules between two liquid phases. An example of a two-phase liquid system is oil and water. In TLC, the solvent is called the **mobile phase**. In partition TLC, there are molecular layers of fluid associated with the adsorbent surface. This fluid represents the **stationary phase**. Molecules to be separated will have a preferred solubility distribution between the mobile and stationary phases depending on their structure. Molecules that have little or no solubility in the mobile phase and high solubility in the stationary phase will migrate slowly. Conversely, sample molecules that have little solubility in the stationary phase but have high solubility in the mobile phase migrate the fastest.

Partition TLC plates are made of cellulose where the stationary phase consists of water molecules hydrating the cellulose fibers. This hydration water does not behave like free water and is analogous to a gel-like phase. In almost all cases, both partition and adsorption processes operate simultaneously in the same TLC experiment. For instance, certain pigment molecules could directly interact with the cellulose fibers and would be involved in an adsorption process.

OVERVIEW OF THE EXPERIMENT

Pigments travel at different rates because they are not equally soluble in the solvent. The chlorophylls are only slightly soluble in water. These molecules consist mostly of hydrogen and carbon, with some groups containing nitrogen and oxygen. The magnesium atom is tightly bonded to the nitrogen's in a central cavity. Chlorophyll *a*, located in the reaction center of photosystems, is the primary photosynthetic pigment in plants. Chlorophyll 'a' and 'b' are structurally identical except chlorophyll 'a' contains a methyl group (-CH₃), while 'b' contains an aldehyde group (-CHO). The aldehyde makes chlorophyll 'b' more polar and slightly more soluble in polar solvents, such as ethanol and water, than chlorophyll 'a'. β-carotene has very low polarity since it consists only of hydrogen and carbon. Vitamin A is a derivative of

Background Information

β -carotene. Xanthophyll has a very similar structure to β -carotene, but also contains two hydroxyl groups (-OH) that are polar and make xanthophyll and its derivatives considerably more water and alcohol soluble than the chlorophylls and β -carotene. The chloroplast pigments are generally prepared by extracting plant leaves with organic solvents such as ether and acetone which are much less polar than water. For example, ether is not soluble in water.

This laboratory consists of **two** experiments. In the **first experiment**, a plant pigment mixture is prepared and resolved into its major components by cellulose TLC. Pigments from leaf tissue (spinach) are disrupted by the use of extraction buffer and are extracted from their protein-membrane complexes. The pigments preferentially partition into the organic phase, forming a mixture making possible the separation of the mixture by TLC. The pigments from spinach leaves should not be extracted by blending in ether or acetone. This procedure is dangerous because of the extremely flammable nature of these solvents. A spark from the electric motor of the blender can violently ignite the solvents. EDVOTEK® has developed an alternate solvent which avoids the use of ether or acetone systems to produce concentrated pigment samples. EDVOTEK® has developed a safe TLC solvent which primarily contains a mixture of alcohols that is safe. It will separate the mixture of β -carotene, chlorophyll a, chlorophyll b and xanthophylls. The Rf values when using this solvent will vary from the results obtained for that where either ether or acetone solvent is used.

The **second experiment** involves the assay of the light reactions of photosynthesis using intact chloroplasts and the dye DPIP. DPIP will substitute for NADP⁺ as the electron acceptor in chloroplast preparations. DPIP is blue in its oxidized state and becomes colorless when reduced. Since intact chloroplasts produce reducing equivalents (electrons) when exposed to light, the assay preparation will lose its blue color with time. A spectrophotometer is used to measure the resultant increase in light transmittance over a period to time.

Chloroplasts are fragile and remain most active if kept in ice cold, isotonic, buffered solutions. The ionic detergent, sarkosyl, denatures proteins and dissolves biological membranes. Solutions containing sarkosyl will disrupt chloroplast structure and destroy the photosynthetic process. This reagent will be used as a more convenient alternative to boiling which destroys chloroplast activity as a control for the photosynthesis assay.



Experiment Overview

EXPERIMENT OBJECTIVE

1. To understand how thin layer chromatography separates a mixture of plant pigments.
2. To understand and calculate Rf values of plant pigments.
3. To demonstrate light dependent reactions of photosynthesis by colorimetric assay.
4. To gain an understanding of the relationship between light wavelength, light intensity and photosynthetic rate.

WORKING HYPOTHESIS

1. If leaf tissue consists of a number of different pigments, then chromatography can be used to separate and identify them.
2. If living chloroplasts and light energy are necessary requirements of photosynthesis, then intact chloroplasts exposed to light produce electrons that are accepted by DPIP resulting in a decrease in color intensity as DPIP is reduced and the percent of light transmittance will increase with time.
3. If living chloroplasts and light energy are necessary requirements of photosynthesis, then chloroplasts in either an ionic detergent or dark conditions will result in a decrease in photosynthesis, DPIP will not be reduced, and the percent of light transmittance will stay the same over time.



Wear gloves
and safety
goggles

LABORATORY SAFETY

Gloves and safety goggles should be worn routinely as good laboratory practice.

Part One: Thin Layer Chromatography of Plant Pigments

IN THIS EXPERIMENT:

- A mixture of chloroplast pigments in isopropanol has been prepared from spinach leaves. The mixture contains chlorophylls, xanthophyll and its derivatives and small amounts of β -carotene. These pigments can be separated from one another on a cellulose thin layer chromatography plate that will be developed in an alcohol-water based solvent system.
 - A standard mixture of pigments containing chlorophylls a and b, xanthophyll, epoxide derivatives of xanthophyll, and β -carotene is also provided for the chromatography to aid in identifying the components of your pigment sample.
 - After the experiment has been run, a wavy line can be observed at the last location of the solvent's leading edge on the white adsorbent plate (unless the solvent was allowed to run all the way up to the top of the plate). This line is called the solvent front.
 - The distance traveled by the sample from the origin divided by the distance traveled by the solvent front from the origin is defined as the **Rf of the particular component**. A sample that does not migrate from the origin has a $R_f = 0$ while one that has not adsorbed at all and has migrated with the front has $R_f = 1$. R_f is a characteristic value for a substance chromatographed on a specific adsorbent in a given solvent system. R_f values can be dramatically changed by changing the solvent system.
1. Measure 1 cm up from a short side (2 cm) of the TLC plate and LIGHTLY draw a straight line across the white, adsorbent side of the plate with a pencil. This is the sample origin line. If you press too hard, you will scrape adsorbent from backing.
 2. Place the end of the capillary pipette nearest a graduation mark, just below surface of the pigment solution in the micro test tube labeled 'Sample'. Watch carefully and allow the liquid to reach a graduation mark, then withdraw the pipette from the tube.
 3. One microliter of the sample will be applied towards the left side of the plate. Hold the pipette vertically and straight. Touch the end of the pipette to the sample origin line until the level of the sample drops to the next graduation mark. Then quickly lift the pipette away from the adsorbent plate.
 4. Using another capillary pipette, withdraw pigment solution from the microtest tube labeled 'Standard' as described in Step 2.
 5. Apply 1 microliter of standard pigments, as described in Step 3, to the right of the sample.
 6. Allow pigments to dry on plate for approximately 3 minutes.

Remember!



The capillary pipets are delicate, handle them carefully.

Each graduation mark on the capillary pipets corresponds to 1 microliter. The total calibrated volume is 5 microliters.

The exact volume withdrawn is not critical as long as the level of pigment is even with a graduation mark. If more than 5 microliters is withdrawn, touch the pipet's tip to a tissue or paper towel just long enough to allow the level of pigment go down to a graduation mark.



Part One: Thin Layer Chromatography of Plant Pigments

Quick Reference:

Since these pigments are sensitive to oxygen, they will eventually fade.

7. Place short edge of plate that is below sample origin line into the beaker containing solvent. Be sure the level of solvent is lower than the samples.
8. Stand the plate up by using the wall of the beaker as a support. Cover the beaker with plastic wrap.
9. Stand the plate in the solvent for approximately 30 to 40 minutes, as time allows.
10. Remove the plate and lay it flat, adsorbent side up, on a paper towel to dry. The plate can be left for several days at room temperature **IN THE DARK**. Identify your plate by marking your group number or initials on the plastic back.
11. Calculate R_f for each of the spots you observe in the sample and standards.
 - Measure from the sample origin line to the center of the spots.
 - The standards used in this separation are, in order starting from the sample origin line, β-carotene, chlorophyll a, chlorophyll b, xanthophyll, and epoxide derivatives of xanthophyll.
 - The chlorophylls will probably be very close to each other ('b' will appear as a partially resolved zone emerging from 'a').

Analysis of Pigment Bands (millimeters)

Band Number	Distance (mm)	Band Color	Pigment Name	R _f Value
1				
2				
3				
4				
5				
6				

Distance Solvent Front Moved ____ (millimeters)

$$R_f = \frac{\text{distance pigment migrated (mm)}}{\text{distance solvent front migrated (mm)}}$$

Part Two: Photosynthesis

Remember!



Tube 4 is wrapped in foil (including covering the top with the foil).

A suspension of active chloroplasts in phosphate buffered sorbitol has been prepared. You will monitor the light dependent reactions of photosynthesis in the chloroplasts by using the dye DPIP:

- This dye will substitute for NADP^+ , the natural substrate that is used at the end of the photosynthetic sequence of oxidation-reduction reactions.
- DPIP is blue in its oxidized form. When the dye is reduced (has received an electron and a proton), it becomes colorless.
- Since photosynthesis normally generates reducing equivalents used for converting NADP^+ to NADPH , the dye can be used as an indicator for the photosynthetic reactions. The absorption of light energy by chlorophyll initiates these reactions.

1. Label five 13 x 100 mm (10 ml) test tubes 1 through 6 at their tops.
2. Wrap Tube 4 in foil.
3. Using a 5 or 10 ml pipet, add 2.5 ml of 'Student Chloroplast Dilution Buffer' to each of the six test tubes.
4. The following chart describes the pipeting of reagents to each of the tubes. Use a designated pipet for each reagent. For example, use the same pipet for only the chloroplasts, and a different pipet only for the DPIP dye, etc.

TUBE	DISTILLED WATER	CHLOROPLASTS	DPIP	SARKOSYL	
1 (blank, no DPIP)	1.3 ml	0.2 ml	-	-	MIX
2 (no chloroplasts)	0.5 ml	-	1 ml	-	MIX
3 (in light)	0.3 ml	0.2 ml	1 ml	-	MIX
4 (in dark)	0.3 ml	0.2 ml	1 ml	-	MIX
5 (in sarkosyl)	-	0.2 ml	1 ml	0.3 ml	MIX

Mix the tubes thoroughly, since the dilution buffer is denser than the other reagents. **Pipet the sarkosyl in Tube 5 after ALL other pipetings have been completed.** If there is an inadvertent cross-contamination of the sarkosyl into the other tubes, it will ruin the experiment. Use 1 ml pipets for volumes of 1 ml or less.



Part Two: Photosynthesis

Experiment Procedure

5. Bring the tubes to the spectrophotometer. The instrument should be set at 605 nm. If you are using a Spectronic 20, zero the instrument as follows:
 - WITHOUT a tube in the instrument, with the cover to the sample chamber CLOSED, adjust the instrument to read 0% transmittance by adjusting the left knob (power switch/zero control).
 - Open the sample chamber and put in Tube 1. Close chamber and adjust the instrument to read 100% transmittance with the right knob (transmittance/absorbance control).

6. Obtain % transmittance values for Tubes 2, 3 and 5. Record your results in the table on Page 14 under 0 time. Quickly remove the foil from Tube 4 and place it in the instrument. Record the reading and after removing the tube quickly wrap it back up in foil.
7. Stand Tubes 2-5 in a beaker of water. Take it to the lab area where lamps or flood lights are set up for the photosynthesis incubation.
8. Place the beaker approximately 8 inches from the light source and note the time.

Depending on the waiting period for the spectrophotometer, try to obtain % transmittance values for Tubes 2-5 at 0, 5, 10, 15 and 20 minutes after exposure of the tubes to direct light as described below.

9. Zero the instrument as described in Step 5 (Tube 1). Use this tube to calibrate the instrument between readings.
10. Bring the beaker to the spectrophotometer area. Wipe Tube 2 and 3 dry and obtain readings. Record the results and the times in the table.
11. Quickly remove the foil from Tube 4, wipe the tube dry and obtain a reading. Record the results and time. Remove the tube and wrap it in foil.
12. Wipe Tube 5 dry, obtain a reading and record. Put Tubes 3-5 into the beaker and return the beaker to the light source.
13. Take additional readings at 5, 10, 15, and 20 minutes, repeating steps 9-11.

Part Two: Photosynthesis

14. Graph the percentage transmittance from tubes 2, 3, 4, 5.

Experiment Procedure

% TRANSMITTANCE					
TUBE	TIME (in minutes)				
0	5	10	15	20	
2 (no chloroplasts)					
3 (in light)					
4 (in dark)					
5 (in sarkosyl)					



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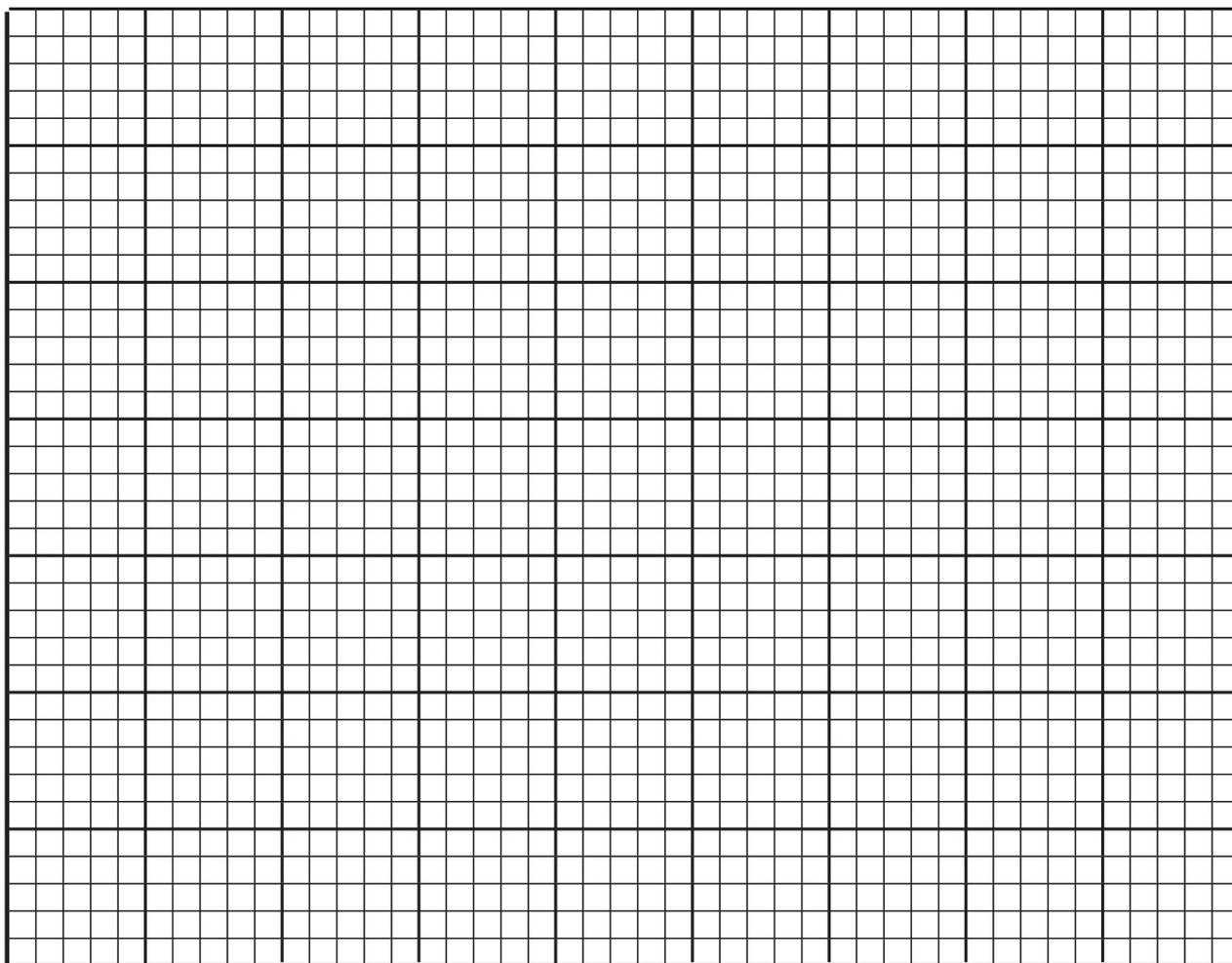
Experiment Analysis

1. Using Graph paper, graph the percent transmittance from Tubes 2, 3, 4, 5 for your lab group.
2. Title the Graph: Percent Transmittance for Chloroplasts Incubated with DPIP versus Time.

What is the independent variable? (x) axis?

What is the dependent variable? (y) axis?

3. What two hypotheses are being tested in this experiment?



Study Questions

1. List and explain some of the factors involved in the separation of pigments?
2. Assuming that partition chromatography was primarily operating in the TLC experiment, which of the four pigments, β -carotene, chlorophyll 'a', chlorophyll 'b' or xanthophyll, is the most soluble in the solvent system used? Which is the least soluble?
3. Which kind of solvent system would you expect to increase the migration rate of β -carotene, water : ethanol (1:1) or petroleum ether : acetone (9:1)? Why? Would you expect the R_f of the carotene to change with these different solvents?
4. If the TLC experiment were run on a longer plate for a longer time, the separation between chlorophyll 'a' and 'b' would become larger. Eventually, two completely resolved spots would be seen. Why? Would you expect the R_f values for the pigments to significantly change between a shorter and a longer TLC run?
5. What is the function of DPIP in the experiment?
6. Explain the purpose of each tube you prepared for the photosynthesis experiment. Did you observe any significant changes in the % transmittance with time in tubes 2-5? What would you expect to occur if tubes 3-5 were moved further away from the light source?
7. Does a decrease in the % transmittance for a colored liquid sample indicate an increase or a decrease in the absorption of the incident light when compared to a colorless reference? If a solution of pigment appears green, does this mean that the light absorbed by the pigment is primarily green? Chloroplasts are too large to go into solution. Aqueous preparations of chloroplasts form suspensions. Suspensions tend to scatter incident light which results in a decrease in the % transmittance. However, this is not the same as absorption of light. When the detergent sarkosyl is added to a suspension of chloroplasts, the % transmittance increases. Why?
8. NADPH absorbs light a 340 nm (ultraviolet region), while NADP⁺ does not absorb at this wavelength. If an ultraviolet spectrophotometer was available, can you design a method to monitor the photosynthetic light reactions with a chloroplast preparation? Assume light scattering will not be a problem.



Notes to the Instructor

OVERVIEW OF LABORATORY INVESTIGATIONS

The "hands-on" laboratory experience is a very important component of the science courses. Laboratory experiment activities allow students to identify assumptions, use critical and logical thinking, and consider alternative explanations, as well as help apply themes and concepts to biological processes.

EDVOTEK® experiments have been designed to provide students the opportunity to learn very important concepts and techniques used by scientists in laboratories conducting biotechnology research. Some of the experimental procedures may have been modified or adapted to minimize equipment requirements and to emphasize safety in the classroom, but do not compromise the educational experience for the student. The experiments have been tested repeatedly to maximize a successful transition from the laboratory to the classroom setting. Furthermore, the experiments allow teachers and students the flexibility to further modify and adapt procedures for laboratory extensions or alternative inquiry-based investigations.

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site.

www.edvotek.com

In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our website:

www.edvotek.com/safety-data-sheets

Pre-Lab Preparations

A. CHLOROPLAST PREPARATION - EXTRACTION OF CONCENTRATED PIGMENTS FOR THIN LAYER CHROMATOGRAPHY

Thaw buffer and reducing stock (components A & B). Refreeze after use. The day before or on the day of the lab:

1. To a clean, 250 ml beaker or flask:
 - Add 80 ml of distilled water .
 - Add 20 ml of chloroplast extraction buffer (A). Mix.
 - Add 1 ml of reducing agent (B).
 - Cover and store in freezer until frozen or partially frozen.
2. Wash fresh spinach in cold tap water. Partially dry with paper towels. Remove stems and midveins from healthy leaves with scissors or knife.
3. Weigh a clean, dry 400 ml beaker (glass or plastic). Approximate to the nearest gram.
4. Cut spinach leaves into small pieces and place into beaker. Prepare 30 grams. Determine leaf mass by weighing beaker and contents and subtracting mass of the empty beaker.
5. Remove diluted chloroplast extraction buffer from freezer and allow it to thaw to an ice slurry (mostly liquid but with some ice left). Place on ice when thawed.
6. Place a clean 250 - 400 ml beaker and 4 plastic, 15 ml centrifuge tubes on ice.
7. Cut 6 pieces of cheesecloth large enough to easily cover the mouth of 250 - 400 ml beaker.
8. Add all (100 ml) of the ice cold, partially thawed diluted chloroplast extraction buffer to a blender.
9. Transfer all the cut leaves to the blender. Cover.
10. Shake blender vessel to wet the leaves.
11. Turn blender on (blend setting) for 10 seconds, then turn off.
12. Repeat Step 11.



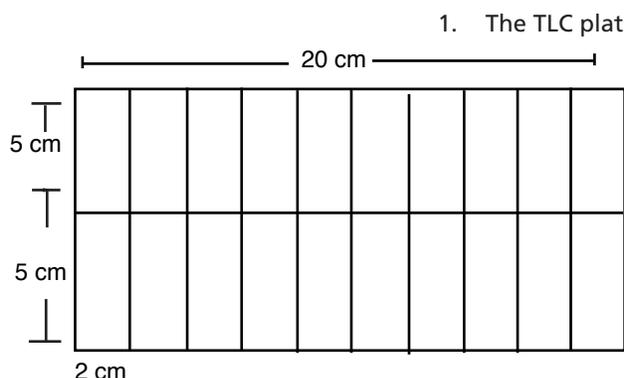
Pre-Lab Preparations

13. Slowly pour spinach homogenate through cheesecloth into ice cold beaker. Periodically stop pouring and allow homogenate to filter through cheesecloth.
14. Occasionally fold up cheesecloth into a sack and squeeze out the residual liquid. Some of the debris may have to be removed from the cheesecloth. Discard the cheesecloth and debris after filtering is complete.
15. Transfer 10 ml of crude extract in the beaker to each of 4 ice cold, plastic centrifuge tubes. Fill to the 10 ml graduation on the tubes. Cap.
16. Place the tubes into a clinical centrifuge. Arrange the tubes so they are properly counterbalanced. Centrifuge at highest speed (approximately 1000 - 2000 x g) for 5 minutes.
17. Pour off supernatant. Blot away residual buffer by keeping the tube inverted on a paper towel for a minute.
18. Using a 1 ml pipet, add 1 ml of 95-100% isopropanol to each tube. Cap.
19. Thoroughly and vigorously mix each tube until the precipitate is broken up and resuspended. Vortex, if possible or use a pipet or stirring rod to help break up precipitate. Liquid should become dark green and thicker.
20. Place the capped tubes in the centrifuge, correctly balanced, and centrifuge at full speed for 10 minutes.
21. Using a plastic transfer pipet, carefully remove the supernatant from each tube and transfer to a plastic microtest tube. Try not to transfer any of the precipitate. You may centrifuge again if necessary.
22. Cap the microtest tubes and label them 'Sample'. Store in the freezer, in the dark.

Pre-Lab Preparations

B. PREPARATION OF THIN LAYER CHROMATOGRAPHY PLATES - ANY TIME BEFORE THE LAB:

Take care not to scrape the adsorbent from its plastic backing. Handle the plate by its edges. Make marks on the white adsorbent side of the plate with a dull pencil.



1. The TLC plate is 10 (length) X 20 (width) cm. Measure up 5 cm on both short sides of the plate. Leave light pencil marks to indicate 5 cm.
2. Bisect the plate horizontally by lightly drawing a line with a blunt pencil and ruler to connect the marks.
3. Lightly make pencil marks every 2 cm on both long edges of the plate.
4. Divide plate vertically 10 times by lightly drawing lines with a blunt pencil and ruler between the 2 cm spaced marks.
5. Using a sharp pair of scissors, cut on the horizontal line (Step 1). Then cut on each vertical line. Do **NOT** use a paper cutter. You should have twenty 5 x 2 cm sections of TLC plates.

Dispensing Reagents

On the day of the lab:

6. Thaw standard pigments (E) and tube containing sample (from Part A).
7. Component E, Standard Pigments, contains isopropanol that may have evaporated over time. Check the volume before dispensing component E for the student groups; there should be 150 μ l (0.15 ml). If there is not 150 μ l of sample, add the appropriate volume of isopropanol to bring the final volume up to 150 μ l.
8. Using a 5 ml pipet, transfer 3 ml of chromatography solvent (F) to each of twenty 100 ml beakers. Cover each beaker with plastic wrap.



Pre-Lab Preparations

9. Using a plastic transfer pipet:
 - Put 2 or 3 drops of sample pigment extract into each of 10 microtest tubes. Cap.
 - Label "Sample".
 - Each tube should be shared between 2 students.
10. Using a fresh plastic transfer pipet, put 1 drop of standard pigments (E) into 10 microtest tubes. Cap.
 - Label "Standard".
 - Each tube should be shared between 2 students

For TLC experiment, each student requires:	For TLC experiment, every two students require:
<ul style="list-style-type: none"> •Chromatography solvent (in covered beaker) •Pencil •One 5 x 2 cm TLC plate •Metric ruler •2 capillary pipets 	<ul style="list-style-type: none"> •1 microtest tube of 'sample' pigment extract •1 microtest tube of 'standard' pigments

Remember!

It is important to keep the chloroplasts ice cold during all stages of preparation and storage. Thaw all buffers.

C. PREPARATION OF CHLOROPLASTS FOR PHOTOSYNTHESIS

It is important to keep the chloroplasts ice cold during all stages of preparation and storage. Thaw all buffers.

On the day before or the day of the lab:

1. To a clean 250 ml beaker or flask:
 - Add 120 ml of distilled water.
 - Add 30 ml of chloroplast extraction buffer (A).
 - Add 1.5 ml of reducing reagent (B). Mix.
 - Transfer 50 ml of diluted extraction buffer to a separate beaker or flask.
 - Cover both vessels. Store the larger volume in the freezer (this is to be used in the blender to homogenize spinach) and the smaller volume in the refrigerator (this is to be used to resuspend chloroplasts).

Refer to pages 18 and 19 and repeat steps 2 - 15 in Pre-lab Preparations, Part A.

2. Place tubes in centrifuge, accurately balanced, and centrifuge at high speed, approximately 1000-2000 x g, for 5 minutes.

Pre-Lab Preparations

3. Pour off the supernatant, blot residual liquid at the mouths of the tubes with a paper towel or wipe. Put the tubes on ice. The pellet may be loose; take care it does not slip out.
4. Remove the vessel containing 50 ml of diluted extraction buffer from the refrigerator and put it on ice.
5. To each centrifuge tube, add 1 ml of diluted extraction buffer.
6. GENTLY resuspend the chloroplast pellets by repetitive pipeting and tapping of the tubes. Keep cold. A clean Pasteur pipet may help to resuspend the chloroplasts.
7. After resuspension, pool all the chloroplast suspensions into one centrifuge tube. Cap.
8. Label "Chloroplast Concentrate". Store on ice in refrigerator.

Within one hour before the lab:

9. Chill a clean 15 ml centrifuge tube on ice.
 - Add 7 ml of distilled water.
 - Add 2 ml of the chloroplast dilution buffer (C).
 - Mix and chill.
10. Chill 6-10 clean 10 ml (13 x 100 mm) test tubes.
 - Gently mix the chloroplast concentrate (Step 9) by tapping.
 - Add 1 ml of the chloroplast concentrate to the tube of cold diluted chloroplast dilution buffer.
 - Cap and gently mix the diluted chloroplasts by inversion. Keep on ice.
 - Dispense 1 ml into each of the chilled test tubes for the student groups.
11. To a clean 400 ml beaker or flask:
 - Add 170 ml of distilled water.
 - Add 80 ml of chloroplast dilution buffer (C). Mix.
 - Label the vessel "Student Chloroplast Dilution Buffer". Store on ice. Dispense 20 ml per group.
12. Prepare an incubation area with several lights.



Pre-Lab Preparations

D. SPECTROPHOTOMETER

On the day of the lab:

The photosynthesis assay is designed so test tubes can be placed directly into a Spectronic 20. If you have another type of instrument with cuvettes, students will need to transfer appropriate volumes for readings. Review operating instructions for spectrophotometer.

1. Warm up the spectrophotometer for one half hour before the lab. Set the wavelength to 605 nm.
2. If a working spectrophotometer is not available the experimental results can be clearly observed after 10 minutes. Similarly, if there is not enough time for all the groups to take readings, visual inspection is sufficient or the number of lab groups can be decreased. However, there are enough reagents for 10 groups.

You may want to time stagger your groups during the Experimental Procedures.

FOR THE PHOTOSYNTHESIS EXPERIMENT, EACH STUDENT GROUP REQUIRES:

- 6-10 clean 10 ml (13 x 100 mm) test tubes
- 100 ml beaker containing approximately 80 ml of water
- Two 5 or 10 ml pipet
- Three 1 ml pipets
- 20 ml of Cold chloroplast dilution buffer on ice
- 5 ml of Distilled water
- 1 ml of Diluted chloroplasts on ice
- 5 ml of DPIP (D)
- 1 ml of Sarkosyl (G)
- Aluminum foil
- Test tube rack
- Pipet pumps

Expected Results - Part One

Analysis of Pigment Bands (millimeters)				
Band Number	Distance (mm)	Band Color	Pigment Name	Rf Value
1				
2				
3				
4				
5				
6				

Typical Rf values for the standard pigments:

EDVOTEK® solvent		Acetone Solvent (not included in kit)		
β-carotene	0	β-carotene	faint yellow	0.98
chlorophyll a	0.29	xanthophyll	reddish yellow	0.80
chlorophyll b	0.44	xanthophyll	yellow	0.50
xanthophyll	0.65	chlorophyll a	blue-green	0.46
		chlorophyll b	yellow-green	0.30
		xanthophyll	yellow	0.15

If using EDVOTEK® solvent, faint yellow spots with Rf values greater than xanthophyll may be observable in the pigment extract sample, Rfs of approximately 0.77 and 0.83. These are epoxide derivatives of xanthophyll, such as lutein epoxide and neoxanthin. The β-carotene may be difficult to detect in the pigment sample extract. When the plate is completely dry, this pigment may be observable as a faint orange-yellow tinge at the origin.

If using acetone as a solvent, β-carotene, a yellow band, should be close to the top of the plate. Several different xanthophylls follow, and range from a yellowish-red and red close to the top, to greys and finally to yellow at around and Rf of 0.50. Close behind this band is a blue-green band, representing an isomer of chlorophyll a, and a yellow-green band at Rf 0.30, representing another isomer of chlorophyll b. The last band which is yellow in color and close to the origin has an Rf of 0.15, is xanthophylls.



Expected Results - Part Two

ANALYSIS OF TUBES

Tube 1 of the photosynthesis experiment should appear green. This tube contains chlorophyll, but no DPIP and is used to calibrate the instrument before and between readings. If all the DPIP was reduced in tubes 3-5, there would still be less than 100% transmittance of light.

Tube 2 should remain dark blue (approximately 1-2% transmittance).

Tube 3 should fade until the % transmittance approaches the value of Tube 2.

Tube 4 and Tube 5 should remain dark blue and give approximately the same % transmittance values as at 0 time (1-3% transmittance). Tube 5 may give somewhat higher initial transmittance readings than tubes 2-4. This is discussed in questions and answers. Typically, Tube 3 gives final transmittance values in the range of 50-60% after 12 to 15 minutes.

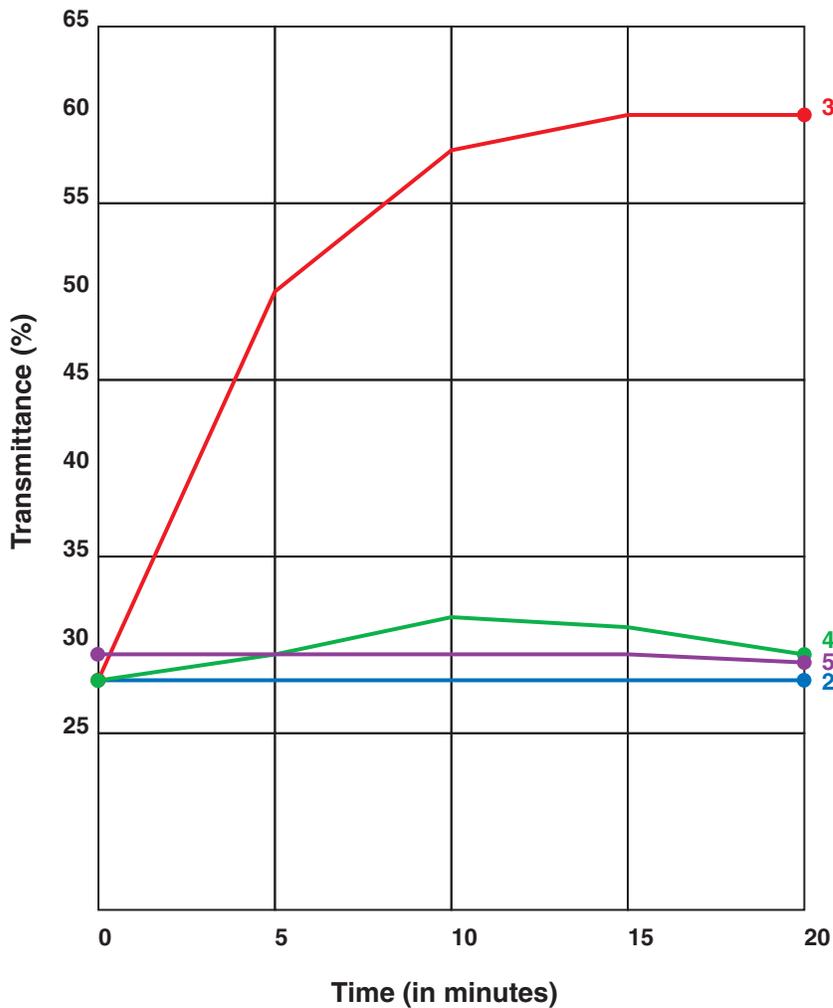
% TRANSMITTANCE					
TUBE	TIME (in minutes)				
0	5	10	15	20	
2 (no chloroplasts)	28.3	28.3	28.3	28.3	28.3
3 (in light)	28.3	50.1	58.5	60.3	60.5
4 (in dark)	28.3	29.5	31.5	31.0	29.5
5 (in sarkosyl)	29.5	29.6	29.7	29.6	29.0

Analysis of Results

1. Graph the percent transmittance from tubes 2, 3, 4, 5 for your lab group.
2. Title: Percent Transmittance for Chloroplasts Incubated with DPIP versus time.

What is the independent variable? (x) axis?
Time in minutes

What is the dependent variable? (y) axis?
Transmittance in percent



3. What two hypotheses are being tested in this experiment?

If intact chloroplasts exposed to light produce electrons that are accepted by DPIP resulting in a decrease in color intensity as it is reduced, then the percentage of transmittance of light will increase with time.

If chloroplasts are absent, in the dark or exposed to sarkosyl, then no electrons will be produced and DPIP will not be reduced (the dark color will remain), then the percentage of transmittance of light will stay the same with time.

KEY

- = no chloroplasts (2)
- = in light (3)
- = in dark (4)
- = in sarkosyl (5)



Answers to Study Questions

- List and explain some of the factors involved in the separation of pigments?**
 - The solvent mixture and the adsorptive surface chosen in order to provide the best separation of the pigments.
 - The affinity of the adsorptive surface and the solubility in the solvent.
 - The time allowed for separation. A short run will result in fewer bands that are not clearly separated from each other.
- Assuming that partition chromatography was primarily operating in the TLC experiment, which of the four pigments, β -carotene, chlorophyll 'a', chlorophyll 'b' or xanthophyll, is the most soluble in the solvent system used? Which is the least soluble?**

Xanthophyll and its derivatives are most soluble (carried furthest by the EDVOTEK® solvent) and β -carotene is the least soluble.
- Which kind of solvent system would you expect to increase the migration rate of β -carotene, water : ethanol (1:1) or petroleum ether : acetone (9:1)? Why? Would you expect the Rf of the carotene to change with these different solvents?**

Petroleum ether-acetone will increase the migration rate because it is much less polar. The β -carotene is not soluble in water and alcohol, but is very soluble in petroleum ether-acetone. The Rf values will be substantially different in the two solvents (for the same adsorbent). Refer to the expected results section for a comparison of migration rates of the two solvents.
- If the TLC experiment were run on a longer plate for a longer time, the separation between chlorophyll 'a' and 'b' would become larger. Eventually, two completely resolved spots would be seen. Why? Would you expect the Rf values for the pigments to significantly change between a shorter and a longer TLC run?**

Samples are able to pass through more adsorbent during longer runs. This increases the amount of fractionation and amplifies small differences between sample components. The Rf values do not significantly change with longer runs because they are constant ratios of sample and solvent front migration distances.
- What is the function of DPIP in the experiment?**

DPIP is a substitute for NADP⁺ in chloroplast preparations. It accepts the electrons removed from the splitting of water in the light dependent phase of photosynthesis. The deep blue color of DPIP will decrease in intensity to colorless as it accepts electrons and is reduced to NADPH.
- Explain the purpose of each tube you prepared for the photosynthesis experiment. Did you observe any significant changes in the % transmittance with time in tubes 2-5? What would you expect to occur if tubes 3-5 were moved further away from the light source?**

Tube 1 is a reference sample to zero the spectrophotometer. It contains chloroplasts, but no DPIP. Tube 2 shows the initial transmittance values due to the dye alone and is a control that can demonstrate whether factors OTHER than the chloroplasts contribute to the dye reduction, i.e., to make sure that only

**Please refer to the kit
insert for the Answers to
Study Questions**